DERIVING A DOSE AND REGIMEN FOR ANTI-GLUCOSAMINIDASE ANTIBODY PASSIVE-IMMUNISATION FOR PATIENTS WITH *STAPHYLOCOCCUS AUREUS* OSTEOMYELITIS

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Abstract

Staphylococcus aureus (*S. aureus*) osteomyelitis remains a major clinical problem. Anti-glucosaminidase (Gmd) antibodies (1C11) are efficacious in prophylactic and therapeutic murine models. Feasibility, safety and pharmacokinetics of 1C11 passive immunisation in sheep and endogenous anti-Gmd levels in osteomyelitis patients were assessed.

3 sheep received a 500 mg intravenous (i.v.) bolus of 1C11 and its levels in sera were determined by enzyme-linked immunosorbent assay (ELISA) over 52 d. A humanised anti-Gmd monoclonal antibody, made by grafting the antigen-binding fragment (Fab) portion of 1C11 onto the fragment crystallisable region (Fc) of human IgG1, was used to make a standard curve of mean fluorescent intensity *versus* concentration of anti-Gmd. Anti-Gmd serum levels were determined in 297 patients with culture-confirmed *S. aureus* osteomyelitis and 40 healthy controls.

No complications or adverse events were associated with the sheep 1C11 i.v. infusion and the estimated circulating half-life of 1C11 was 23.7 d. Endogenous anti-Gmd antibody levels in sera of osteomyelitis patients ranged from < 1 ng/mL to 300 μg/mL, with a mean concentration of 21.7 μg/mL. The estimated circulating half-life of endogenous anti-Gmd antibodies in sera of 12 patients with cured osteomyelitis was 120.4 d.

A clinically relevant administration of anti-Gmd (500 mg i.v. = 7 mg/kg/70 kg human) was safe in sheep. This dose was 8 times more than the endogenous anti-Gmd levels observed in osteomyelitis patients and was predicted to have a half-life of > 3 weeks.

Anti-Gmd passive immunisation has potential to prevent and treat *S. aureus* osteomyelitis. Further clinical development is warranted.

Keywords: Orthopaedic infections, immunoassay, *Staphylococcus aureus*, osteomyelitis, peri-prosthetic joint infection, 2-stage revision surgery, passive immunisation.

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List of Abbreviations			
		Fc	fragment crystallisable region
		FDA	Food and Drug Administration
AAALAC	Assessment and Accreditation of	Gmd	glucosaminidase
	Laboratory Animal Care	IACUC	Institutional Animal Care and Use
Amd	amidase		Committee
Atl	autolysin	ICC	intraclass correlation coefficient
CI	confidence interval	IRB	Institutional Review Board
CV	column volume	i.v.	intravenous
ELISA	enzyme-linked immunosorbent	LLOD	lower limit of detection
	assay	mAb	monoclonal antibody

MFI	median fluorescent intensity
MRSA	methicillin-resistant *Staphylococcus aureus*
PBS	phosphate-buffered saline
PE	phycoerythrin
PJI	peri-prosthetic joint infection
SACs	*Staphylococcus* abscess communities
SCIP	Surgical Care Improvement Project
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
HA	total hip arthroplasty
TKA	total knee arthroplasty
UCB	upper confidence bound

Introduction

Bone infection, also known as osteomyelitis, remains a catastrophic complication of orthopaedic surgery. Most cases are caused by *Staphylococcus aureus* (*S. aureus*) (Darouiche, 2004). Although clinical algorithms have been established to prevent these biofilm infections that are resistant to antibiotic therapy (Saeed *et al.*, 2019), the current incidence of PJI ranges from 0.2 to 1.5 % (Schwarz *et al.*, 2019). Moreover, it appears that significant improvements are not possible, as implementation of the most rigorous protocols [*e.g.* outcomes from the SCIP (Stulberg *et al.*, 2010)] has demonstrated that infection rates for elective surgery cannot be reduced below 1-2 % (Cram *et al.*, 2012). Additionally, on top of the clinical complications, the enormous costs for treating osteomyelitis threaten healthcare systems, as they are projected to exceed $1.62 billion in the USA by 2020 (Kurtz *et al.*, 2012).

The prevalence of *S. aureus* osteomyelitis is due to the various pathogenic mechanisms that this commensal pathogen has evolved to facilitate immune evasion, including:

- biofilm formation on the implant (Nishitani *et al.*, 2015b) and necrotic bone (Birt *et al.*, 2017; Lew and Waldvogel, 2004);
- generation of SACs in soft tissues and bone marrow (Cheng *et al.*, 2009; Varrone *et al.*, 2014; Yokogawa *et al.*, 2018);
- ability to colonise the osteocytic-canalicular network of live cortical bone (de Mesy Bentley *et al.*, 2018; de Mesy Bentley *et al.*, 2017).

Thus, reinfection rates following surgery for *S. aureus* osteomyelitis are very high (15-40 %) and often require an implant-exchange surgery to remedy the problem (Azzam *et al.*, 2009; Ferry *et al.*, 2009; Ghanem *et al.*, 2009; Parvizi *et al.*, 2009; Salgado *et al.*, 2007).

Although active immunisations are the most cost-effective interventions for infectious diseases, enormous efforts to develop a *S. aureus* vaccine have been unsuccessful for various reasons (Jansen *et al.*, 2013; Proctor, 2012; Proctor, 2015). Moreover, active vaccinations targeting endogenous pathobionts such as *S. aureus* may boost pre-existing immune responses, which may be ineffective or may even have debilitating effects. These failures have led to conflict in the field with regard to the role of humoral immunity during *S. aureus* infections and reservations about active vaccination therapy's potential to treat serious surgical site infections (Bagnoli *et al.*, 2012; Fowler and Proctor, 2014; Pier, 2013; Proctor, 2012; Projan *et al.*, 2006). This raging controversy has been amplified by the occurrence of patient deaths in a large phase II clinical trial of the V710 vaccine (Fowler *et al.*, 2013; McNeely *et al.*, 2014), posing the question of how the extensive pre-clinical research programme leading up to a clinical trial with 8,000 patients could have reached the wrong conclusions regarding the vaccine safety and efficacy for the intended clinical indication (Harro *et al.*, 2012; Kim *et al.*, 2010). However, this pessimism is tempered by the recent transformative successes of cancer mAb-based passive immunotherapies after decades of disappointments, for which the 2018 Nobel Prize in Physiology or Medicine was awarded to Dr James Allison and Dr Tasuku Honjo for their seminal discoveries of immune checkpoint inhibitors (Smyth and Teng, 2018). Thus, the quest for an *S. aureus* vaccine continues.

The overall hypothesis of the passive immunotherapy programme has been that the most effective mAbs would have dual-acting mechanisms of action, both directly inhibiting functions critical to *S. aureus* and mediating immunomodulatory activity to boost host response and bacterial clearance. By using a non-biased research approach to test this hypothesis, the Gmd subunit of *S. aureus* Atl was identified as the lead target (Gedbjerg *et al.*, 2013; Varrone *et al.*, 2014; Varrone *et al.*, 2011b; Yokogawa *et al.*, 2018). Of note is that other groups have also identified Atl as an immunodominant antigen (Brady *et al.*, 2011; Gotz *et al.*, 2014; Holtfreter *et al.*, 2010). Functionally, Atl is essential for cell-wall degradation and biosynthesis during binary fission (Oshida *et al.*, 1995; Sugai *et al.*, 1995; Yamada *et al.*, 1996). In addition, Atl acts as an adhesin (Heilmann *et al.*, 2005), a biofilm enzyme (Brady *et al.*, 2006) and a facilitator of host cellular internalisation/immune evasion (Hirschhausen *et al.*, 2010). Remarkably, *S. aureus* Amd, which is the other subunit of Atl, known to activate platelet activation and aggregation (Binsker *et al.*, 2018), was shown to be a molecular target of vancomycin (Eirich *et al.*, 2011), which is the most common antibiotic used to treat MRSA infections. Importantly, anti-Gmd passive immunisation has been shown to synergise with vancomycin therapy in rabbit and murine infection models (Brady *et al.*, 2011; Kalali *et al.*, 2019; Yokogawa *et al.*, 2018). Additionally, clinical research studies to assess humoral immunity in patients with osteomyelitis from PJI, trauma and diabetic foot ulcers have identified anti-Gmd antibodies in patients that recover from these serious infections (Gedbjerg *et al.*, 2013; Nishitani *et al.*, 2015a; Oh *et al.*, 2018).

To evaluate the potential of anti-Gmd mAb passive immunisation in murine models of osteomyelitis, 36 anti-Gmd-producing murine hybridomas were screened for their ability to bind recombinant Gmd, inhibit its enzymatic activity in a cell-wall digestion assay and precipitate *S. aureus* out of culture (Gedbjerg *et al.*, 2013; Varrone *et al.*, 2014; Varrone *et al.*, 2011a). Of these, one IgG1 mAb (1C11) displayed superior properties based on its:

- clinically relevant affinity ($k_f = 3.1 \times 10^4$ M^{-1}s^{-1}; $k_r = 5.0 \times 10^{-5}$s^{-1}; $K_D = 1.6 \times 10^{-9}$ M) (Varrone *et al.*, 2014);
- stoichiometric neutralising activity (Gedbjerg *et al.*, 2013);
- phenocopy of Gmd deficient *S. aureus* mutants (Varrone *et al.*, 2014);
- ability to mediate *S. aureus* megacluster formation and opsonophagocytosis *in vitro* (Varrone *et al.*, 2014);
- ability to protect mice from MRSA osteomyelitis (8 out of 17 animals demonstrated undetectable MRSA levels in the 1C11 group as compared to 1 out of 15 in the placebo group) (Varrone *et al.*, 2014);
- ability to synergise with vancomycin to cure mice with established osteomyelitis [combination therapy yielded a 6.5-fold reduction in MRSA levels (7 out of 10 animals) as compared to untreated animals] (Yokogawa *et al.*, 2018).

Although 1C11 does not inhibit bacteria within biofilms, these promising results warrant further research to transform the established rodent dosing regimen (40 mg/kg through intraperitoneal injection) to that of FDA-approved biologics (*e.g.* infliximab Remicade®, trastuzumab Herceptin®, rituximab Rituxan®, which are chimaeric mAb) administered as an i.v. infusion in the order of 500 mg/L saline (7 mg/kg for a 70 kg patient). Clinical research is also needed to assess endogenous anti-Gmd levels in osteomyelitis patients. Thus, 1C11 passive immunisation of sheep was evaluated and the concentration of circulating anti-Gmd antibodies in patients with *S. aureus* osteomyelitis was quantified.

Materials and Methods

Large scale production of 1C11 mAb

A pilot study to assess feasibility, safety and pharmacokinetics of passively immunising adult sheep was performed at the AO Research Institute (Davos, Switzerland) based on IACUC-approved protocols. To generate the test material, 2 g of 1C11 mAb were produced from a well-characterised 1C11 hybridoma cell line (Gedbjerg *et al.*, 2013; Varrone *et al.*, 2014) in the Upstate Stem cGMP Facility (USCGF, University of Rochester, Rochester, NY, USA). Following adaptation to HyClone SFM4Mab medium with 1 % foetal bovine serum (Thermo Fisher Scientific), an initial 1 L inoculum of 1C11 hybridoma cells (1.5×10^5 viable cells/mL,

1C11 = 18.6 µg/mL) was placed in a 5 L Cellbag™ for WAVE Bioreactor™ (GE Healthcare Life Sciences) at 37 °C. As the viable cell count reached $\sim 8.2 \times 10^5$ cells/mL (1C11 = 56.8 µg/mL), an additional 1.5 L of 1 % serum-containing medium was added. Following dilution of the culture with additional medium, the viable cell count expanded to $\sim 1 \times 10^6$ cells/mL (1C11 = 109.2 µg/mL). At that point, 2 L of culture were harvested and 2 L of 1 % serum medium was added to replace the volume in the Cellbag™. As the cell population increased (1.8×10^6 cells/mL, 33 µg/mL), an additional 2 L of culture was harvested and 1 L of serum-free medium added, bringing the total serum concentration to approximately 0.2 %. As the cell count remained stationary at $\sim 7 \times 10^5$ cells/mL, another 1.5 L of 0.2 % serum medium was added. After an initial lag in cell growth, the population of 1C11 hybridoma cells resumed growth and 2 L of product were harvested, followed by the addition of 1 L of serum-free medium, bringing the effective medium serum concentration to 0 %. At this point, 1C11 cells (3.9×10^5 cells/mL) were fully adapted to serum-free medium. With minimal lag, cells reached a cell count of 1.09×10^6 cells/mL (45.3 µg/mL), so an additional 1 L serum-free medium was added, followed shortly thereafter with harvesting of the entire 2 L content of the Cellbag™ (1.6×10^6 cells/mL, 74.4 µg/mL). In total, 8 L of product were obtained from the entire Cellbag™ adaptation run, with an estimated antibody production of ~ 90 µg/mL. The 8 L of culture were harvested in 4 independent 2 L harvests. The culture was processed by microfiltration using a Millipore filtration system with Pellicon® filter (0.22 µm Durapore® microfiltration membrane) to separate the cells, yielding ~ 2 L of clarified culture supernatant. The clarified culture supernatant was subsequently concentrated ~ 5.7-fold to a final volume of ~ 350 mL using a 30 kDa molecular weight cut-off ultrafiltration membrane. The concentrate was aliquoted and stored at -20 °C.

Purification of the 1C11 mAb

A 60 mL aliquot of concentrated culture supernatant (~ 30 mg of 1C11 mAb) was thawed and loaded directly onto a MAbSelect SuRE LX Protein A column (GE Healthcare Life Sciences) equilibrated in 25 mM NaPO$_4$/0.15 M NaCl (pH = 7.2) using an ÄKTAprime chromatography system (GE Healthcare Life Sciences) and a flow rate of 2.5 mL/min. Post loading and washing of the column with equilibration buffer (pH = 7.2), 60 mL of the 1C11 antibody culture supernatant were again added to the column. Following loading, the unbound protein was washed through the column with five CVs (1 CV = 5 mL) of equilibration buffer. Following column wash, elution of the 1C11 product was accomplished by application of a linear gradient (0-0.1 M) of sodium citrate (pH = 3.0). Elution of the bound 1C11 mAb was effected with a pH gradient from 7.2 to 3.0 over 10 CVs (2.5 mL fractions were collected). Fig. 1 describes the results of a typical run

in which ~ 30.8 mg of 1C11 antibody in concentrated culture supernatant was loaded onto the column and ~ 23.0 mg of 99 % pure mAb were recovered, resulting in an overall yield of 74.7 %. This process was repeated until 2 g of 1C11 antibody were purified. The protein integrity was confirmed by SDS-PAGE stained with Coomassie Brilliant Blue according to manufacturer's recommendations (BioRad). The antigen binding activity of the purified 1C11 antibody was demonstrated by ELISA

Passive immunisation of sheep and pharmacokinetic assessment in sera

Three adult (2 years), healthy (based on clinical examination and blood analysis) Swiss Alpine female sheep weighing 56-57.5 kg were acclimatised for 2 weeks. During this time, as well as after the infusion, they were group-housed and fed twice per day with hay and mineral lick at their disposal. Each sheep was given a 500 mg i.v. bolus of mouse IgG1 1C11 mAb in 1 L of Ringer's solution over a period of 3 h. During infusion, sheep's physiological parameters were observed by a veterinarian. These included heart rate, respiration rate, temperature and anaphylactic reaction. Post infusion, sheep were monitored daily for the first 10 d and weekly thereafter using a customised score sheet (temperature increases, potential infusion site infections, weight gain/loss and faeces changes). Blood was withdrawn for haematological analyses including complete blood
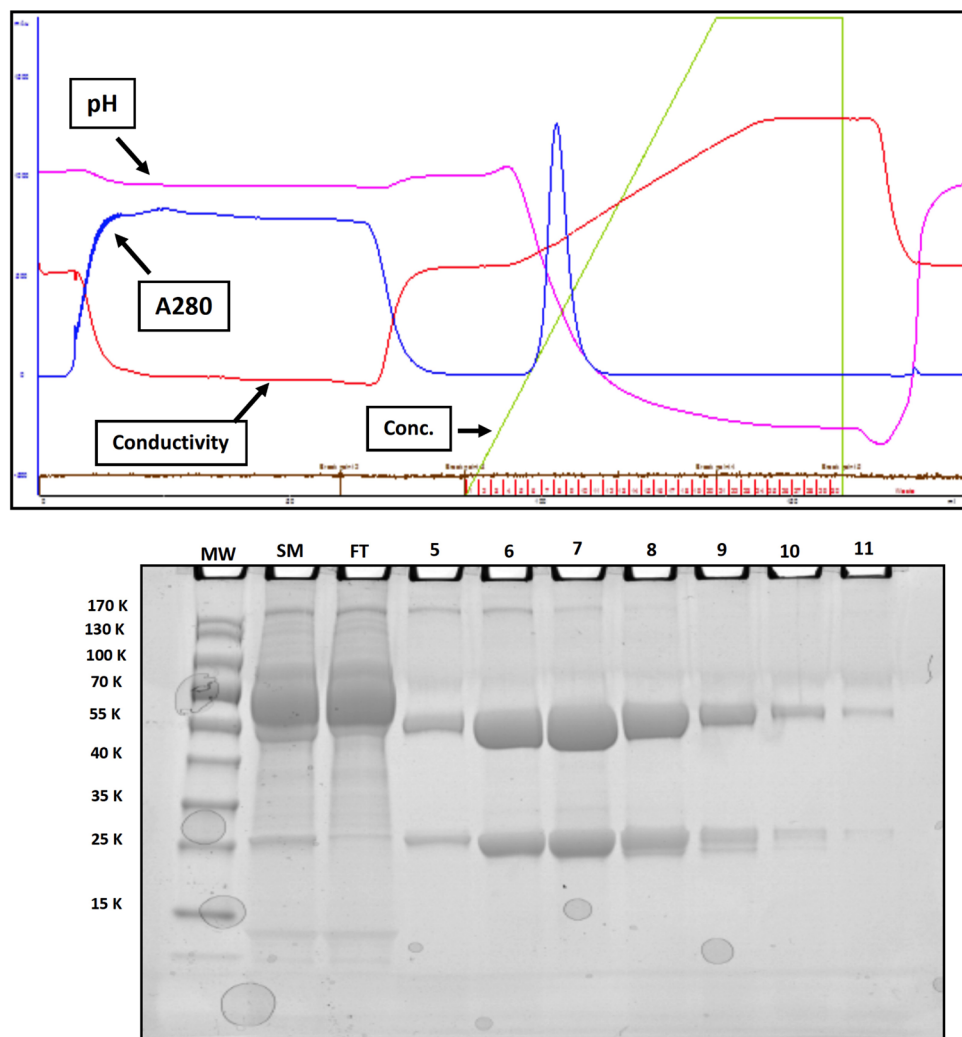

Fig. 1. Large scale purification of 1C11 mAb. Large scale protein purification of 1C11 mAb (~ 30 mg in 60 mL of concentrated hybridoma culture supernatant) was performed using an ÄKTAprime chromatography system as described in Materials and Methods. Biochemistry data on a representative run are shown to illustrate the process and the purity and integrity of the resulting product. (Top) Real-time data acquisition of the eluate was performed to assess pH, protein concentration (A280), chemoelectrical conductivity and sodium citrate elution buffer during the chromatography run. Results are presented in an overlaid graph format. Of note is the sharp elution of the antibody (narrow blue peak of A280 midway through the run), which corresponds to the 2.5 mL elution fractions 6 to 9. (Bottom) An image of a Coomassie Brilliant Blue-stained denatured SDS-PAGE gel of molecular weight standard (MW), starting material (SM), flow through (FT) and elution fractions 5-11 is shown to illustrate the relative purification of the heavy (53 kDa) and light (25 kDa) chains of the 1C11 mAb. In this run, 30.8 mg of protein was loaded on to the column and 23.0 mg of 99 % pure mAb was recovered, giving a 74.7 % yield.

count, total protein, serum electrolytes, blood urea nitrogen and creatinine before and after infusion as well as twice per week thereafter.

Additionally, blood was taken 1 h post infusion (to determine C_0) daily for 2 weeks and then weekly for 2 months. Serum was prepared from each blood sample and stored frozen at − 20 °C until all samples were collected. Then, 2 mL aliquots of the entire collection were assayed for anti-Gmd antibody activity by sandwich ELISA using recombinant histidine-tagged Gmd protein for capture and horseradish-peroxidase-conjugated anti-murine IgG1 antibody for detection, as previously described (Varrone *et al.*, 2014).

All *in vivo* work was carried out at the AO Research Institute Davos, Switzerland, in an AAALAC-International-approved facility and according to the Swiss animal protection law and regulations (approval number 05_2013).

Assessment of anti-Gmd antibody levels in human sera by Luminex assay

All human-subject research was performed following IRB-approved protocols. A worldwide clinical registry of 297 patients with culture-confirmed *S. aureus* osteomyelitis was established in 2013 and completed in 2018 (Kates *et al.*, 2019). This registry contains three serum samples (0 month, diagnosis of infected implant; 6 months, re-implantation; 12 months, follow up) from each patient. For the present study, anti-Gmd antibody titers were determined from the 297 patients of the AO5 registry and 40 uninfected healthy controls. To quantify the relative concentration of anti-Gmd antibodies in the sera, a mouse-human chimaeric 1C11 mAb was generated by replacing the murine Fc with the human Fc IgG1, while retaining the murine V region
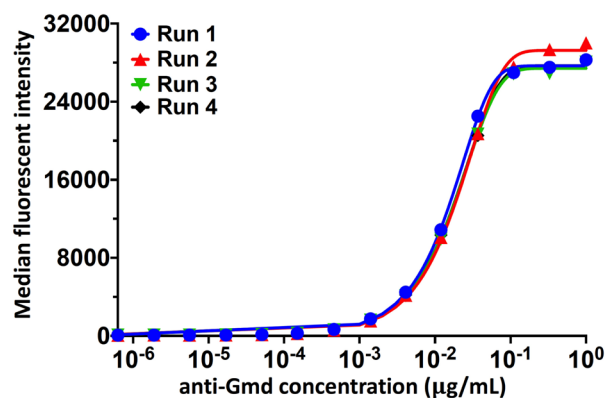

Fig. 2. Standard curves of the humanised 1C11 chimaeric anti-Gmd mAb. Standard curves of the MFI *vs.* concentration of the chimaeric 1C11 anti-Gmd antibody were generated using Luminex immunoassay starting at 1 µg/mL and serially diluting 3-fold down 14 times. This experiment was performed 4 independent times (n = 4) and the data from the 4 runs are superimposed in the graph. Highly significant reproducibility was achieved between the experiments, as illustrated by an ICC of 0.9984 [95 % CI: 0.9964-0.9994, p = 1e⁻²³].

of the mAb, using a proprietary method (US Patent 9,683,054; BioAtla Inc., San Diego, CA, USA). Thus, this chimaeric mAb had the same antigen-binding characteristics as 1C11 but could be recognised by the anti-human IgG secondary antibody used in the Luminex assay.

The Luminex assay was performed as previously described (Nishitani *et al.*, 2015a). Briefly, 6.5 µm avidin-coated magnetic beads (MagPlex-Avidin Microspheres, Luminex Corporation, Austin, TX, USA) were coupled to recombinant biotinylated glucosaminidase protein (GenScript USA Inc., Piscataway, NJ, USA). A standard curve was generated with the chimaeric 1C11 mAb, in which the starting concentration of 1 µg/mL was serially diluted 3-fold 14 times. The mAb was incubated with 1,000 Gmd-coupled magnetic microspheres per well of a Luminex immunoassay 96-well plate. After a 2 h incubation, the plate was washed using an automatic plate washer (BioTek 405TS microplate washer, BioTek Instruments, Inc., Winooski, VT, USA), 100 µL of the secondary PE-conjugated anti-human IgG (Southern Biotech, Birmingham, AL, USA) were added to each well and the plate was incubated for 1 h. Duplicate samples were analysed on a flow cytometer (Luminex 200, Luminex Corporation) to generate the standard curve of anti-Gmd titer MFI *versus* concentration of chimaeric anti-Gmd mAb (µg/mL) (Fig. 2). The aggregate LLOD for anti-Gmd antibody titers was utilised to define the threshold of detection. LLOD was calculated using the formula LLOD = MFI of assay buffer + 2× SEM of assay buffer MFI (Nishitani *et al.*, 2015a; Oh *et al.*, 2018). An MFI of 95 when projected on the anti-Gmd 1C11 standard curve corresponded to 0.0139 ng/mL. Thus, although Luminex assays typically have detection thresholds in the pg/mL range, similar to that found with the mAb, a more conservative approach was used to set the limit of detection at 1 ng/mL, which took the large dilution factor (1 : 10,000) into consideration. Therefore, the values measured were the true anti-Gmd 1C11 levels in serum of patients with *S. aureus* osteomyelitis. The experiment was performed 4 independent times and highly significant reproducibility was achieved between the experiments, as illustrated by an ICC of 0.9984 [95 % CI: 0.9964-0.9994, p = 1e⁻²³].

To determine the anti-Gmd antibody titers in human sera, the same Luminex assay was performed by incubating 100 µL of 1 : 10,000 diluted serum in PBS with the Gmd-MagPlex-Avidin Microspheres and then the secondary PE-conjugated anti-human IgG. MFI values were interpolated from the chimaeric 1C11 standard curve considering the 1 : 10,000 dilution factor to determine the concentration of anti-Gmd antibody in each serum sample.

Data analyses

Decay curves to determine antibody half-life of 1C11 in sheep (n = 3) were generated in GraphPad Prism version 8.0, in which the mean concentration at each time point was used to generate the best fit curve and

the time at which 50 % of the C_0 was observed was determined to be the circulating antibody half-life. To determine the proportion of AO5 sera with an anti-Gmd concentration above uninfected control levels, UCB analyses were performed in which the 95 % UCB for the control group was used as a threshold.

Results

Circulating half-life of murine 1C11 anti-Gmd mAb in sheep

Based on the demonstration of the safety and efficacy of 1C11 anti-Gmd passive immunisation in prophylactic (Varrone *et al.*, 2014) and therapeutic (Yokogawa *et al.*, 2018) models of implant-associated osteomyelitis, the present study aimed to establish preliminary safety and pharmacokinetic data in sheep, whose total body mass (~ 56 kg) and dosing capacity (~ 500 mg of mAb in 1 L of saline, i.v.) are similar to humans. Thus, 2 g of 1C11 mAb were manufactured and administered to 3 adult sheep in a single i.v. infusion. Circulating levels of murine IgG1 (1C11 mAb) were assessed over the next 52 d (Fig. 3). There were no complications or adverse events associated with the 1C11 mAb passive immunisation in any of the sheep and the serology results demonstrated a steady decay with a half-life of ~ 23.7 d. Thus, this dosing regimen was deemed to be safe and appropriate for assessment of 1C11 mAb efficacy in a sheep model of implant-associated osteomyelitis (Moriarty *et al.*, 2017).

An additional consideration for treatment of at-risk patients with prophylactic anti-Gmd mAb 1C11 is the likelihood that treatment will elicit a potentially neutralising sheep anti-mouse (or anti-human) response. To address this concern, the abundance of sheep anti-mouse IgG (heavy and light chain) antibody present in the serum of each sheep was measured at the time of administration (day 0) and 4 weeks later (day 28). Expectedly, no evidence for emergence of an anti-mouse response was found at this relatively early time point (data not shown).

Circulating levels and natural decay of endogenous anti-Gmd antibodies in patients with *S. aureus* osteomyelitis

To support a human dose and regimen of anti-Gmd mAb passive immunisation as an adjuvant therapy for *S. aureus* osteomyelitis, naturally occurring levels were assessed in these patients and healthy controls. Fig. 4 shows the relative concentration of anti-Gmd antibodies in the serum of 297 patients at the time of their revision surgery (active infection), compared to the mean concentration of anti-Gmd in sera of 40 uninfected healthy controls. Anti-Gmd antibody concentrations in the patient sera ranged from undetectable (< 1 ng/mL) to 300 µg/mL. The concentration of anti-Gmd in the median patient was 21.7 µg/mL, which was within the range of uninfected control sera (25.6 ± 28.6 µg/mL). Interestingly, only 104 (35.0 %) patients had circulating anti-Gmd antibody levels significantly above the mean of healthy people ($p < 0.05$ using a 95 % UCB = 33.2 µg/mL), suggesting that most *S. aureus* osteomyelitis patients failed to develop an immune response against this antigen and that passive-immunisation with a protective anti-Gmd mAb was indicated for a large majority of these patients.

To gain insight into a clinically relevant dosing regimen of anti-Gmd mAb immunotherapy for chronic osteomyelitis, for which the standard of care is 6 to 8 weeks of i.v. antibiotic therapy (Masters *et al.*, 2019), the decay of circulating anti-Gmd antibodies was assessed within this patient cohort, over their 1-year treatment period. There were two major prerequisites for inclusion: 1) the patient completed the 1-year follow up period, 2) the patient was
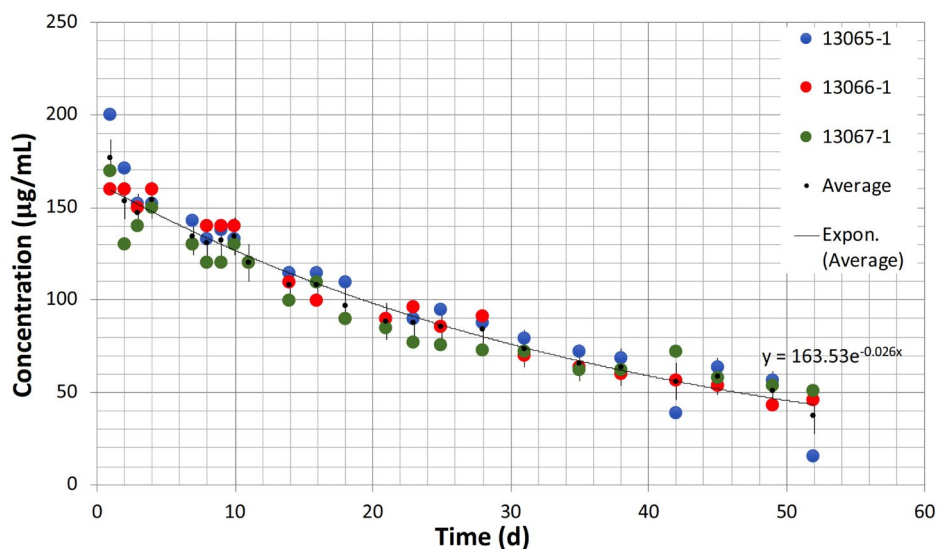

Fig. 3. Half-life of murine mAb 1C11 in sheep. To determine basic pharmacokinetic parameters of candidate mouse mAbs in sheep, 3 candidate sheep were injected with murine 1C11 and the blood (serum) was collected for 52 d to measure the concentration of mouse IgG1. Fitting the averaged data to a single decay curve yielded an estimated circulating half-life of about 23.7 d.

clinically cured of *S. aureus* osteomyelitis. Fig. 5 shows the results of a *post-hoc* analysis that identified 12 patients whose immune proteome and clinical profile was consistent with a cured *S. aureus* osteomyelitis, in that they had > 20 µg/mL of anti-Gmd antibodies in their serum prior to treatment and displayed no clinical signs or symptoms of infection following treatment. Additionally, their circulating anti-Gmd antibody titers markedly decreased from baseline to 6-month time point. Thus, assuming insignificant

endogenous anti-Gmd antibody synthesis following treatment, the mean half-life of anti-Gmd antibodies in this cohort was 120.4 d, suggesting an approximately 3-month dosing regimen for passive-immunisation with a protective anti-Gmd mAb for this indication. Nonetheless, the primary intention of the anti-Gmd therapy is a one-time preventative treatment in patients lacking anti-Gmd antibodies that are about to undergo TKA/THA.
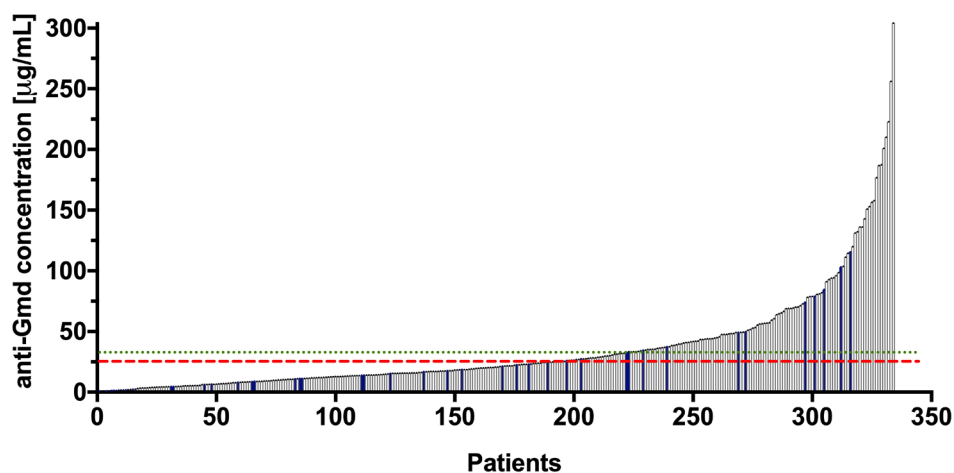

Fig. 4. Naturally occurring anti-Gmd antibody levels in serum from a large cohort of patients with *S. aureus* osteomyelitis. Serum was obtained from 297 patients with culture-confirmed *S. aureus* osteomyelitis (represented in black) and 40 healthy individuals (represented in blue) with no reported infections. The concentration of anti-Gmd antibodies in each sample was determined by Luminex assay as described in Fig. 2. The anti-Gmd concentrations (µg/mL) are presented in rank order from the lowest to the highest and ranged from undetectable (< 1 ng/mL) to 304 µg/mL. The concentration of anti-Gmd in the median patient was 21.7 µg/mL. The red dashed line indicates the mean concentration of anti-Gmd (25.6 ± 28.6 µg/mL) in sera of 40 uninfected healthy controls and the green dotted line is the 95 % UCB = 33.2 µg/mL.
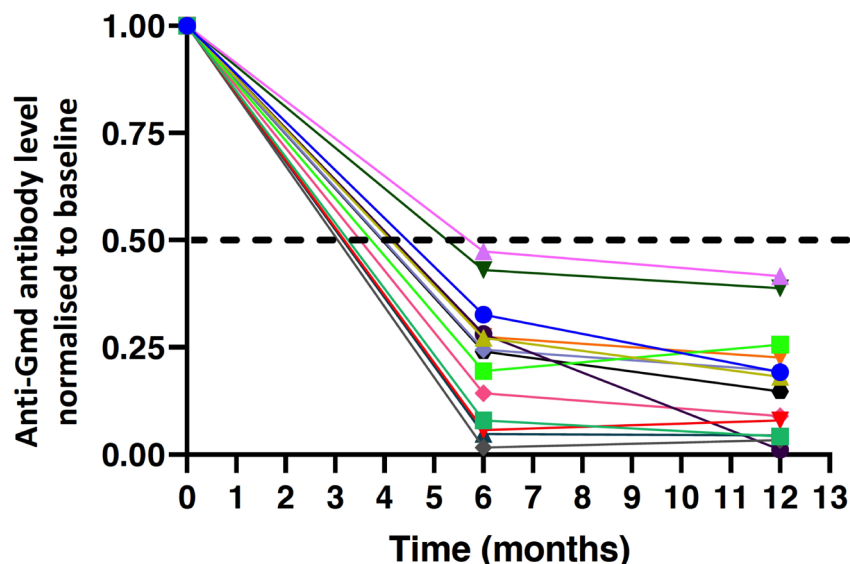

Fig. 5. Analysis of endogenous anti-Gmd antibody level decay in patients with cured *S. aureus* osteomyelitis. A *post-hoc* analysis was performed on the *S. aureus*-infected cohort to identify a cured sub-group that had > 20 µg/mL of anti-Gmd antibodies in their serum prior to treatment and who displayed no clinical signs or symptoms of infection following treatment. Anti-Gmd antibody levels in the sera of the 12 patients identified in the cured sub-group were determined by Luminex assay and normalised values based on each patient's anti-Gmd antibody level at 0 months (baseline) are presented. Assuming insignificant endogenous anti-Gmd antibody synthesis following treatment, the mean half-life of anti-Gmd antibodies in the cohort was 120.4 d.

Discussion

An effective immunotherapy against the primary pathogen responsible for most musculoskeletal infections would be transformative for orthopaedic surgery. Unfortunately, none exists and a major contributing factor to failures in *S. aureus* vaccine development has been the absence of an *in vivo* model with face and construct validity of surgical site infections (Reizner *et al.*, 2014; Salgado-Pabon and Schlievert, 2014). Thus, the approach to develop a passive immunisation has focused on murine models with quantitative outcomes of *in vivo* planktonic growth, biofilm bacteria on the implants, SACs, invasion and colonisation of the osteocytic-canalicular network of cortical bone, osteolysis and implant osseointegration (de Mesy Bentley *et al.*, 2017; Inzana *et al.*, 2015; Li *et al.*, 2008; Nishitani *et al.*, 2015b; Varrone *et al.*, 2014; Yokogawa *et al.*, 2018). Also, a sheep model of a failed two-stage revision of intramedullary-nail-related infection by MRSA was developed (Moriarty *et al.*, 2017), which is a suitable model for testing passive immunisation.

Concerning the vaccine's molecular mechanism of action, the study hypothesis was that an ideal passive immunotherapy would be an mAb with both direct antimicrobial effects through inhibition of a critical *S. aureus* target and immunomodulatory activity to enhance host response and bacterial clearance. From a non-biased antigen discovery, *in vitro*, animal model and clinical research point of view, Gmd was identified as a validated target for immunotherapy (Gedbjerg *et al.*, 2013; Nishitani *et al.*, 2015a; Oh *et al.*, 2018; Varrone *et al.*, 2014; Varrone *et al.*, 2011b). Additionally, a lead anti-Gmd mAb (1C11) was developed over 36 candidates, based on its superior *in vitro* characteristics (Gedbjerg *et al.*, 2013; Nishitani *et al.*, 2015a; Oh *et al.*, 2018; Varrone *et al.*, 2014; Varrone *et al.*, 2011b) and its safety and efficacy in prophylactic and therapeutic murine models of implant-associated MRSA osteomyelitis (Varrone *et al.*, 2014; Yokogawa *et al.*, 2018). Remarkably, the results show that 1C11 synergises with the standard-of-care antibiotic therapy (vancomycin) in the 1-stage exchange model of MRSA through distinct mechanisms of actions, as vancomycin decreases the bacterial burden on the implant, but only anti-Gmd mAb inhibits SACs (Yokogawa *et al.*, 2018). Thus, given its potential as an adjuvant therapy for PJI, the study aimed to further substantiate the feasibility of anti-Gmd mAb passive immunisation by: 1) demonstrating safety and favourable pharmacokinetics following a clinically relevant dose in sheep, 2) defining serum levels of anti-Gmd antibodies in patients with *S. aureus* osteomyelitis.

From a feasibility standpoint it is important to note that mAb therapies have been broadly adopted into virtually all areas of medicine and that maturity of this form of biological therapy has recently evolved to generic drugs, known as biosimilars (Ishii-Watabe and Kuwabara, 2019). While subcutaneous mAb therapies

exist (Adalimumab, Denosumab, Secukinumab, *etc.*), most mAb drugs were initially approved as i.v. formulations, partly due to the higher fidelity of this form of dosing regimen for study in clinical trials. Moreover, the 1st generation chimaeric mAb therapies (*e.g.* infliximab Remicade®, trastuzumab Herceptin®, rituximab Rituxan®) are still administered to patients this way. Based on the well-established formulation of these mAbs, which broadly conform to an i.v. infusion of ~ 500 mg/L saline (7 mg/kg for a 70 kg patient), this bolus dose was evaluated in sheep, whose mass is ~ 75 % of that of a human. An important initial finding of this experiment was the ease of manufacturing 1C11 as a drug (Fig. 1) and its transport frozen from Rochester, New York, USA to Davos, Switzerland, without significant loss of material or potency (no significant mAb degradation or aggregation). While the absence of any adverse events during and 52 d following the i.v. infusion of 500 mg of murine 1C11 mAb into the 3 sheep was not surprising, the 23.7 d circulating half-life of the mAb in this xenogeneic host (Fig. 3) was beyond expectations based on immunogenicity concerns. However, those findings in a very small sample size (*n* = 3) cannot be generalised and this simple proof-of-concept study was not meant to circumvent the very rigorous preclinical safety and toxicology studies required to justify the use of an anti-Gmd mAb in human patients.

A major safety advantage of anti-Gmd mAb over biologics that target host factors (*e.g.* anti-cytokine mAb) is that the mAb binds to a bacterial gene product that does not exist in humans. Thus, the potential off-target effects of this mAb are very limited and Gmd is expected to be highly immunogenic in people, as recombinant Gmd protein is highly immunogenic in experimental animals grown in germ-free environments (Brady *et al.*, 2011; Varrone *et al.*, 2011a). A major theoretical concern with anti-Gmd mAb passive immunisation was that it would be superfluous to the patient's endogenous anti-Gmd antibodies. Therefore, the remarkable finding that 65 % of patients with active *S. aureus* osteomyelitis failed to generate circulating anti-Gmd IgG titers above that of healthy control sera (Fig. 4) and commercially available i.v. IgG (Rongsheng® Human Immunoglobulin, pH 4, i.v. injection, 50 g/L, Chengdu Rongsheng Pharmaceuticals Co., Ltd. Chengdu, Sichuan, China. Intravenous immunoglobulin contains 38.36 µg/mL of anti-Gmd antibody, data not shown) suggested that these patients cannot generate humoral immunity against this critical antigen on their own and that anti-Gmd passive immunisation was indicated for them. While this conclusion was based on the calculations of endogenous anti-Gmd levels in human sera that were validated by Luminex assay, it should be noted that a limitation of the approach was the use of a single purified mAb (1C11) to quantify polyclonal anti-Gmd antibodies in human sera.

To better understand anti-Gmd immunity, as a future direction, the functionality of the anti-Gmd antibodies from the patients with very high titers (> 100 µg/mL,

n = 23, Fig. 4) has to be assessed, as the non-protective effect of these anti-Gmd antibodies could be due to their inability to neutralise Gmd enzymatic activity or to contain an inappropriate Fc (*i.e.* IgG4) that is incapable of fixing complement and/or mediating opsonophagocytosis by activated leukocytes. Lastly, as the standard-of-care antibiotic therapy for chronic *S. aureus* osteomyelitis is given over long periods (months) and definitive cure cannot be established with less than 6-weeks of treatment, the study aimed to gain insight on the number of anti-Gmd mAb doses needed during the 1-year treatment period following confirmed *S. aureus* osteomyelitis by determining the decay of endogenous anti-Gmd antibodies in patients with a cured phenotype (Fig. 5). Although the calculated half-life of 120.4 d was an overestimate, as it unreasonably assumed no *de novo* anti-Gmd antibody production following the baseline blood draw and autogenous antibodies have higher stability than biologics, the 3 to 4 infusions per year that this predicts is consistent with cancer immunotherapies (ipilimumab Yervoy®, pembrolizumab Keytruda®, nivolumab Opdivo®) (Schwarz *et al.*, 2019). Although more stringent experiments could be performed, formal assessments of biodistribution and circulating anti-Gmd mAb half-life are not warranted until a drug for clinical trials is available.

Conclusions

Based on its dual mechanisms of action that includes direct antimicrobial effects and immunomodulation, anti-Gmd mAb passive immunisation has emerged as a potential prophylaxis for patients at high-risk of surgical-site infections and as an adjuvant to antibiotic therapy for *S. aureus* osteomyelitis. Towards clinical trials, the present study established that an anti-Gmd mAb had favourable drug manufacturing and storage characteristics and that a clinically relevant dose (8.9 mg/kg/i.v.) was safe in sheep. Anti-Gmd mAb therapy was warranted for the majority of patients with active *S. aureus* osteomyelitis who failed to develop humoral immunity against Gmd.

Acknowledgements

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Discussion with Reviewer

Kunt Ohlsen: Why have you selected sheep for the pharmacokinetics study?
Authors: The researchers of the AO Research Institute are experts in sheep models, particularly for orthopaedic implants. With that in mind, we felt that sheep would be a reasonable model for antibody prophylaxis of implant-associated infections. Also, sheep are similar to humans in weight.

Kunt Ohlsen: How relevant are sheep as compared to other animal models?
Authors: Sheep and goats are now considered to be the primary large animal models for orthopaedic trauma studies based on ethical concerns in using canine and non-human primates, their similar mass to humans and cost-effectiveness.

Kunt Ohlsen: What is known on the anti-Gmd antibodies found in patients surviving osteomyelitis?
Authors: We are currently characterising the anti-Gmd antibodies in these patients and intend to publish the physical bind properties and Gmd inhibition data in a subsequent publication, together with IgG4 anti-Gmd antibody titers. We are also preparing a publication focused on correlating the serology with clinical outcomes of the entire cohort.

Kunt Ohlsen: Why do the authors think that a humanisation is not necessary since osteomyelitis treatment is longer than any other type of infection? **Authors**: While we agree that a fully human anti-Gmd mAb would be better, there are FDA-approved chimaeric antibodies that are used to treat life-long diseases (*e.g.* infliximab for Crohn's disease and rheumatoid arthritis). Thus, it is established that humanisation of chimaeric mAb is not necessary for long-term treatments.

Editor's note: The Scientific Editor responsible for this paper was Mauro Alini.